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DNA extraction, 16S rRNA gene amplification and sequencing

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An abbreviated version of this protocol was published in eLIFE in Aug 2017

Regulation of life span by the gut microbiota in the short-lived African turquoise killifish

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Detailed protocol

A protocol for taxonomic profiling of fecal and intestinal microbiota in the African turquoise killifish (*Nothobranchius furzeri*)

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Abstract:
Killifish are emerging as a new laboratory system to study a range of questions, from the genetic basis of embryo dormancy (Hu *et al*., 2020) to life history trait evolution (Cui *et al*., 2019; Willemsen *et al*., 2020), age-dependent neurodegeneration (Di Cicco *et al*., 2011; Matsui *et al*., 2019; Tozzini *et al*., 2012; Van Houcke *et al*., 2021), as well as the connection between microbial community structure and biology of aging (Smith *et al*., 2017). Here we optimized a protocol for 16S rRNA amplicon sequencing in gut tissue and fecal pellets from laboratory turquoise killifish. We used this protocol to study gut microbiota composition in natural killifish populations, as well as in laboratory killifish. Here we provide a detailed protocol to collect samples, extract DNA, amplify and sequence the V3V4 (as well as the V4) region of the 16S rRNA gene.

Background:
Over the past decade, advances in high-throughput sequencing helped uncover the vast diversity of microbial communities present in environmental samples and on host epithelia. Compared to shotgun metagenomics, 16S rRNA amplicon sequencing requires limited sequencing coverage and is commonly used to characterize the microbial taxonomic composition of a large number of samples. In our past work, we leveraged sequencing of the V3V4 region of the 16S rRNA to unravel age-associated community dynamics in the intestinal microbiota of the short-lived vertebrate *Nothobranchius furzeri* (Smith *et al*., 2017). Here, we describe a comprehensive protocol for high-throughput extraction of genomic DNA from intestinal and fecal samples of *Nothobranchius furzeri* and provide step-by-step instructions for the generation of 16S V3V4 rRNA gene libraries. The amplicon size of the V3V4 region is compatible with use of the Illumina MiSeq technology (2x300), while the size of the V4-fragment amplicon is compatible with HiSeq platforms (2x250), which allows for higher sequencing depth. In this protocol, we provide details for sequencing both the V3V4 (for gut and stool) and the V4 region (for stool only).

Materials and reagents
Consumables:

- 1.4 mm Steel beads (BioSpec, Cat. No.: 11079115ss)
- 0.1 mm Zirkonia/silica beads (BioSpec, Cat. No.: 11079101z)
- PCR tubes
- CleanNGS magnetic DNA extraction beads (CleanNA, cngs-0050)
- Filter tips (various sizes)
- PCR plates
- 2 ml screw-cap tubes (Roth, XC83.1)

Reagents:

- MilliQ water, autoclaved
- Lysis buffer: 80 mM EDTA, 200 mM Tris (pH 8.0) and 100 mM NaCl in PBS
- RNAse A (Qiagen, 1007885)
- 20% SDS (panreac, A0675,0250)
- G2 solution (Qiagen, 13888-100-2)

- C2 solution (Qiagen, 12888-100-2)
- C3 solution (Qiagen, 12888-100-3)
- Proteinase K (Thermo Fisher Scientific, EO0492)
- Lysozyme (Sigma-Aldrich, 62970-5G-F)
- DNAzap (Invitrogen, AM9890)
- Ethanol
- EDTA (Invitrogen, AM9261)
- Tris-base/Trizma (Sigma-Aldrich, T2691-1L)
- Phosphate Buffered Saline 10x (Invitrogen, AM9625)
- Inhibitor Removal Solution (Qiagen, 26000-50-2)
- tricaine/MS-222 (Sigma-Aldrich, A5040-250G)
- RNAlater (Sigma-Aldrich, R0901-100ML)

Recipes:

- Lysis buffer (sterile filtered):

80 mM EDTA (pH 8.0)
200 mM Tris (pH 8.0)
100 mM NaCl
1x Phosphate Buffered Saline

Oligonucleotides:

Table 1. Overview of 16S rRNA primer. Depending on the target region, either the 341F/805R primer pair or the updated 515F/806R primer pair was used. A staggering pad of 6-8 random nucleotides can be added to the primer to increase nucleotide diversity during sequencing.

Targetregion	Primer Name	Sequence	Reference
V3V4	341F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTACGGGNGGCWGCAG	(Caporaso *et al*., 2011)
	805R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGACTACHVGGGTATCTAATCC	(Caporaso *et al*., 2011)
V4	515F	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGYCAGCMGCCGCGGTAA	(Parada *et al*., 2016)
	806R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCGGACTACNVGGGTWTCTAAT	(Apprill *et al*., 2015)

Equipment

Note: No specific equipment is required. Any appropriate device can be used.

- Scale
- Pipettes
- TissueLyser II (Qiagen)
- Centrifuge
- PCR tube magnet
- PCR thermocycler
- Vortexer
- Bench-top centrifuge
- Horizontal gel-electrophoresis system
- Qubit 2.0 with DNA high-sensitivity kit (Invitrogen, Q32851)
- TapeStation with D1000 tape (Agilent, 5067-5582) and reagents (Agilent, 5067-5583)

Procedure

Fecal sampling and dissections

A. *Dissection to harvest the entire intestine*
   1. Euthanize fish in tricaine (1.5 g/L circulatory system water) for about 3-5 minutes.
   2. Open the fish ventrally from anus to the head region.
   3. Make two small lateral incisions and lift the skin to expose the inner organs.
   4. Carefully cut the intestine right posterior to the cranium and at the rectum.
   5. Free the intestine of associated fat tissue and remove the gal bladder.
   6. Cut [illegible]

6. Snap-freeze intestines in liquid nitrogen and store at -80 °C.

A. *Collection of fresh fecal samples*

1. Disinfect a clean tank (2.8 L, Aquaneering) with 70% ethanol and fill with 1 L autoclaved water from the circulatory system.
2. Carefully transfer the fish from its home tank into the collection set-up.
3. Check regularly and collect the feces into 750 ul RNAlater using a pipette with 1000 ul filter tips.
4. At the end of the collection (~6 hours), place the fish back into its original tank.
5. Centrifuge the samples at 4 °C, 15000 rcf for 5 minutes.
6. Remove the supernatant and elute pellet in 750 ul fresh RNAlater for storage at -80 °C.

DNA extraction

1. **Preparation**

1. Sterilize the silica beads by incubation in HCl and subsequent washing with sterile-filtered, autoclaved milliQ-water until the pH is neutral. Then autoclave the beads and sterilize under UV-light.
2. Sterilize the steel beads under UV light.
3. Prepare 2 ml screw-cap tubes with 0.1 g of silica beads and 15-20 steel beads.
4. Add 300 ul lysis buffer to the 2 ml screw-cap tubes.
5. Pre-cool the bead-beating adapters at -20 °C.

1. **DNA isolation**

1. Sterilize all the handling equipment with 70% ethanol, UV-light and DNAZap; and the bench with DNAzap.
2. Add Lysozyme freshly to the lysis buffer on the day of DNA isolation (to a final concentration of 20 mg/ml).
3. In order to dissolve the lysozyme, warm the buffer to 37 °C for 10 minutes.
4. Thaw the fecal or intestinal samples on ice.
    1. For feces: Using a P1000 pipette tip, pick the fecal pellets from the RNAlater solution into the prepared bead-beating tubes.
    2. For intestines: Transfer the intestine into the prepared bead-beating tubes using sterilized forceps.
5. Bead-beat the samples for 2 x 3 minutes at 30 Hz.
6. Spin the samples down for 5 minutes at 15 °C and 8000 rcf.
7. Transfer 78 uL of the supernatant to PCR tubes containing 2 uL RNAse A. The remaining supernatant can be stored at -20 °C for later DNA isolation.
8. Vortex shortly followed by a 30-minute incubation at 55 °C.
9. Add 10 uL Proteinase K and 10 uL 20% SDS.
10. Vortex the samples for 1 minute followed by a 1-hour incubation at 55 °C.
11. Add 40 uL of C2 solution, mix by pipetting and incubate 5 minutes at 4 °C.
12. Spin down the samples for 1 minute.
13. Transfer 100 uL into new PCR tubes containing 35 uL C3 solution. Mix by pipetting and incubate for 5 minutes at 4 °C.
14. Spin down the samples for 1 minute.
15. Transfer 100 uL of the supernatant to new PCR tubes.
16. Add 100 uL of pre-warmed CleanNGS beads and incubate for 10 minutes.
17. Transfer to a magnet, wait for 5 minutes until beads are drawn aside.
18. Remove the supernatant and wash twice with 150 uL of 80% Ethanol (freshly prepared) for 30 seconds.
19. Dry the beads for 5-10 minutes, then remove the tubes from the magnet and elute the beads in DNAse/RNAse free water. The elution volume is dependent on tissue size and desired concentration. We generally use 20 uL for fecal samples and 30-50 uL for intestinal samples.
20. Quantify the DNA concentration on a Qubit 2.0 fluorometer (Invitrogen, DNA HS assay kit).

Library preparation

1. Set up a 25 uL PCR reaction for the extracted DNA samples according to Table 2.

Table 2. Setup of reactions for the first PCR reaction.

	Intestine V3V4	**Feces V3V4**	**Feces V4**
Sample type:	intestine	feces	feces
Target region:	V3V4	V3V4	V4
DNA input:	±50 ng	1-5 ng	1-5 ng
Forward primer:	1 ul 341F (10 uM)	1 ul 341F (10 uM)	1 ul 515F (10 uM)

Reverse primer	1 ul 805R (10 uM)	1 ul 805R (10 uM)	1 ul 806R (10 uM)
KAPA HiFi HotStart ReadyMix	12.5 ul	12.5 ul	12.5 ul
DNAse/RNAse-free water	adj. 25ul	adj. 25ul	adj. 25ul
Number of cycle:	30 cycles	26 cycle	26 cyle

1. The cycling conditions for the PCR amplification are: 98 °C for 5 minutes, followed by 30 (for intestines) or 26 cycles (feces) of 98 °C for 30 seconds, 61 °C for 30 seconds, 72 °C for 30 seconds, with a final elongation phase for 5 minutes at 72 °C.
2. Clean-up the PCR reaction following the bead-based approach described under DNA isolation (use 30 uL of beads). Elute the samples in 30 uL DNAse/RNAse free water.
3. Set up a 25 uL PCR reaction according to Table 3:

Table 3. Setup of reactions for the second PCR reaction.

DNA input:	7.5 ul of cleaned PCR1 product
Forward primer:	2.5 uL i5/P1 primer (10 uM)
Reverse primer	2.5 uL i7/P2 primer (10 uM)
KAPA HiFi HotStart ReadyMix	12.5 ul

1. The cycling conditions for the PCR amplification are: 98 °C for 5 minutes, followed by 8 cycles of 98 °C for 30 seconds, 61 °C for 30 seconds, 72 °C for 30 seconds, with a final elongation phase for 5 minutes at 72 °C.
2. Clean-up the PCR reaction following the bead-based approach described under DNA isolation (use 30 uL of beads). Elute the samples in 30 uL DNAse/RNAse free water.
3. Load 5-20 µl of the PCR reaction on a 1.5% (w/v) agarose gel including 1x RotiSafe DNA gel stain in 1x TAE buffer. Amplicons of ~430 bp and ~610 bp are expected for V4 or V3V4 libraries, respectively.
4. Quantify the libraries with the Qubit 2.0 fluorometer (Thermo Fisher Scientific, DNA HS assay kit).
5. Pool the library in equimolar ratios.
6. Confirm the library quality with TapeStation (Agilent, D1000 tape).
7. Sequence V3V4 libraries with the MiSeq Reagent Kit v3 (600 cycles). The shorter V4 libraries can also be sequenced using the HiSeq Rapid SBS Kit V2 (500 cycles) or with the MiSeq Reagent Nano Kit v2 (500 cycles).

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